Abstract. Background: The main goal of this study was to evaluate the diagnostic efficacy of melanoma-associated antigen (MAGE) A1-6 reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) of bronchial washing fluid for the detection of lung cancer invisible by bronchoscopy. Patients and Methods: To determine the expression of MAGE A1-6 gene in 75 lung carcinomas diagnosed by conventional fluoroscopy-guided lung biopsy and 58 cancer-free controls, RT-nested PCR was performed of bronchial washing fluid. MAGE A1-6 RT-nested PCR data was analyzed according to tumor histology, stage, size, and compared with cytological data. Results: MAGE A1-6 RT-nested PCR displayed higher sensitivity (64.0%) than that of conventional cytology (14.7%). There was no significant correlation between MAGE gene expression and histological types or clinical stage. For tumor size, detection rates were 74.0% in tumor smaller than 3 cm and 58.7% in these larger than 3 cm. Conclusions: MAGE A1-6 RT-nested PCR of bronchial washing fluid may be a useful method for diagnosis of peripheral lung cancer in clinical practice.

Computed tomography (CT) scans can detect non-calcified peripheral located pulmonary lesions that may represent lung cancer, and these lesions require more accurate procedures to differentiate between those that are malignant and those that are benign. CT provides very good anatomical information. However, its most important shortcoming is its poor specificity because of the high prevalence of benign lesions (1).

Imaging-guided transthoracic needle biopsy (TTNB) is a commonly accepted and effective tool for the pathologic diagnosis of peripheral pulmonary tumors. The accuracy and sensitivity rate of TTNB are high but TTNB also has some complications, such as pneumothorax (2, 3). Therefore, new noninvasive or less invasive diagnostic approaches that can differentiate benign lesions from lung cancer prior to TTNB are necessary. We tested a new marker, melanoma-associated antigen (MAGE) gene that is commonly expressed in lung cancer and various types of tumors (4-6), but not in any normal tissue except testis and placenta (7, 8). MAGE A1-6 Common primer is enable the detection of MAGE A1 to A6 subtypes simultaneously. We hypothesized that MAGE A1-6 gene detection by reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) would be useful as a biomarker to detect peripheral lung cancer. The main goal of this study was to prospectively apply MAGE RT-nested PCR in the routine clinical diagnosis procedure and evaluate its diagnostic efficacy using bronchial washing fluid for the detection of lung tumors located beyond the visible range of the bronchoscope.

Patients and Methods

Study subjects. Between March 2007 and February 2008, patients with peripheral lung lesions suspected as lung cancer were recruited in this prospective study. The eligible subjects were 160 patients. Patients with a previous history of lung cancer or malignant disease of other organs were excluded. All patients underwent bronchoscopy and diagnostic thoracic CT scan. CT scans were reviewed prior to bronchoscopy. Bronchoscopy was always performed before TTNB or surgical lung biopsy. Final diagnosis was obtained by pathologic and bacteriologic examinations. Cancer-free individuals (n=85) were used as controls. Cancer-free patients were followed up with serial radiographic imaging at least 24 months to confirm their pulmonary condition. This study was approved by Yeungnam University Medical Center Institutional Review Board and all patients gave their informed consent.

Clinical diagnosis of lung cancer and benign diseases. For most patients, histology was obtained by conventional fluoroscopy-guided

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TTNB. Patients with histological findings considered positive for malignant cells or highly suspicious for malignancy were classified as lung cancer. If the result of biopsy showed a benign entity such as abscess, granuloma, or hamartoma, the diagnosis was usually regarded as reliable. Even if patients were clinically diagnosed with cancer-free status or the initial specimen was inadequate, the growth rate of the pulmonary lesion was evaluated for at least 24 months. A negative result was considered a true negative if i) there was surgical confirmation, ii) the lesion disappeared or decreased in size, or iii) the lesion remained stable as seen on follow-up for at least 24 months.

Collection and processing of bronchial washing fluid. Bronchial washing specimens were collected through a 5.8 mm flexible fiberoptic bronchoscope (Pentax, Tokyo, Japan). Bronchial washing fluid was obtained by instilling 20 ml of normal saline into a tumor-bearing segmental bronchus and retrieving fluid after three to five breaths. This specimen was divided into two portions: one portion was sent for conventional cytology and the other was used for MAGE A1-6 RT-nested PCR. All specimens were examined by one pathologist and reported according to predetermined classification as unsatisfactory, normal, atypia, suspicious of cancer, cancer (9).

MAGE A1-6 RT-nested PCR in bronchial washing fluid. Guanidine isothiocyanate buffer was added immediately to bronchial washing fluid at a ratio of 5:1 for better RNA preservation. The mixed samples were blindly sent to the laboratory and stored in a -70°C freezer until RNA extraction. RNA was extracted using mRNA extraction kit based on the magnetic bead method (iC&G Co., Daegu, South Korea). RNA purities and concentrations were measured by spectrophotometry (DU530, Beckman, CA, USA), then 1 μg of RNA was used for reverse transcription using ImProm-II® enzyme (Promega, Madison, WI, USA). To maximize cancer detection, MAGE was amplified with MAGE capture® Kit (iC&G Co., Daegu, South Korea) using MAGE A1-6 common primers as described by Park et al. (10). The MAGE products were amplified with 30 cycles of nested PCR, the products were run for 30 minutes in a Mupid electrophoresis chamber (Advance, Tokyo, Japan) using 1.2% agarose gel. Glyceraldehyde-3-phosphate-dehydrogenase (GAPD) PCR system was included in the same kit reagents.

Statistical analysis. Database editing and statistical analysis were carried out using SPSS ver. 15.0 software (SPSS Inc., Chicago, IL, USA). The Pearson’s chi-square test and Fisher’s exact test were used to compare positive rates of conventional cytology and MAGE A1-6 RT-nested PCR. Linear by linear association chi-square was used to compare positive rates of conventional cytology and MAGE A1-6 RT-nested PCR. All specimens were examined by one pathologist and reported according to predetermined classification as unsatisfactory, normal, atypia, suspicious of cancer, cancer (9).

Results

Patient characteristics. Clinical characteristics of the eligible participants are summarized in Table I. Of the 160 subjects, 66 were diagnosed with lung cancer by conventional fluoroscopy-guided TTNB and one patient by surgical biopsy. Among the remaining 93 patients, the tumor size gradually increased in eight patients. These patients later underwent biopsy and lung cancer was also confirmed by conventional fluoroscopy-guided TTNB. The majority of patients had lung adenocarcinoma. The median age of lung cancer patients was 65 years and 69.3% were males. Among patients with non-small cell lung cancer (NSCLC), tumors in the majority of patients staged III or IV (61%).

Of the 85 cancer-free individuals, 27 were excluded due to incomplete follow-up, of less than 24 months. The remaining 58 were mostly case with tuberculosis or tuberculosis. Diagnoses made in 40 patients by TTNB, in two patients by surgical biopsy, and in 16 patients by bacteriologic study.

Diagnostic accuracy: overall diagnostic value and comparison of the conventional cytology and MAGE A1-6 RT-nested PCR. MAGE RT-nested PCR was successfully performed in all bronchial washing fluid obtained from the cancer and cancer-free patients. The sensitivity of MAGE A1-6 RT-nested PCR was significantly higher than that of conventional cytology (64.0% vs. 14.7%, p<0.001). Of 75 lung cancer patients, false-negative result was found 27 cases. Twelve false-positive results were seen with this method. The positive and negative predictive values for MAGE A1-6 RT-nested PCR were 80.0%, and 63.0%, respectively. Furthermore, combining the
two methods produced an increased sensitivity of 72% in the diagnosis of lung cancer not accessible with bronchoscopy, although there was no statistically significant difference \( (p=0.293) \) (Table II).

Comparison of detection rates according to histopathology, the stage of NSCLC and the tumor size. MAGE mRNA was detected in 64.9% of adenocarcinomas, 55.6% of squamous cell carcinomas, and 87.5% of small cell lung carcinomas. For all tumor histologies, the sensitivity of MAGE A1-6 RT-nested PCR was greatly higher than that for conventional cytology. The detection rate of MAGE A1-6 RT-nested PCR was relatively high for adenocarcinoma and small cell lung carcinoma. However, there was no difference in MAGE expression between tumor histologies \( (p=0.436) \). Although not statistically significant, combining both MAGE A1-6 RT-PCR and conventional cytology showed increased sensitivity for adenocarcinoma and squamous cell carcinoma (Table III).

When the clinical stage of the patients was considered, there was no difference in MAGE expression \( (p=0.765) \). The detection rate for stage I and II patients was 60% and 75%. These were also not different from those with stage III and IV \( (I \text{ and II vs. III and IV}, p=0.789) \). The detection rate of MAGE A1-6 RT-nested PCR was not different with respect to tumor size \( (p=0.185) \). Nonetheless, the detection rates of MAGE A1-6 RT-nested PCR tended to be increased for tumors smaller than 3 cm sized (Table III).

False-positive rate of MAGE A1-6 RT-nested PCR. Twelve cases (20.7%) of benign disease among 58 individuals were positive by MAGE A1-6 RT-nested PCR. In particular, MAGE mRNA was expressed relatively highly in chronic granulomatous diseases, such as tuberculosis and benign granuloma. Hence, the specificity of MAGE A1-6 RT-nested PCR was 79.3% (Table IV).

Discussion

In this study, we demonstrated that MAGE A1-6 RT-nested PCR is a promising diagnostic method for the detection of lung cancer not visible with bronchoscopy. Considering the invasiveness and complication of TTNB and the test characteristics of MAGE RT-nested PCR, MAGE A1-6 RT-nested PCR of bronchial washing specimens might provide an alternative option to establish diagnosis of peripheral pulmonary lesion.

In normal somatic tissue, methylation of CpG islands within gene promoter is responsible for gene silencing (11).
MAGE is activated by promoter demethylation in cancer of different histologic origin (12). In lung cancer, many researchers reported the expression rate of MAGE using single or two subtypes of MAGE and their expression rate was reported mostly to show only 30-50% of lung cancer tissue (13, 14). Most studies have indicated a high expression rate of MAGE-A3 (15) except one study (16). With the use of membrane array method, Tsai et al. have discovered 88% of MAGE-A2 gene expression in NSCLC tissue (16). Individual members of the MAGE family are frequently expressed in lung cancer but the expression rates are variable and low. In order to increase MAGE detection rates, some researchers have developed multi-marker RT-PCR. We have used multiple MAGE-recognizing primers, that can bind to the sequences of cDNA of MAGE-A1, -2, -3, -4a, -4b, -5a, -5b, and -6 (MAGE-A1-6) simultaneously and detect the specific expression of at least one gene (10). By using this common primer, lung cancer detection rates were 83.3% in the cancer tissue, 70.0% in bronchial washing fluid, and 47.5% in random sputum (17). Recently, there was the first report for the detection rate of MAGE-A1-6 RT-nested PCR in bronchial washing fluid of peripheral lung cancer compare with CT-guided TTNB (18). Kim et al. reported that MAGE RT-nested PCR showed a dramatic increase of sensitivity (67.9%) compared with that of cytology (21.4%). No difference in the sensitivity between MAGE RT-nested PCR (67.9%) and TTNB (73.1%) was reported (18). With the use of membrane array method, Tsai et al. discovered 88% of MAGE-A2 gene expression in NSCLC tissue (16). Individual members of the MAGE family are frequently expressed in lung cancer but the expression rates are variable and low. In order to increase MAGE detection rates, some researchers have developed multi-marker RT-PCR. We have used multiple MAGE-recognizing primers, that can bind to the sequences of cDNA of MAGE-A1, -2, -3, -4a, -4b, -5a, -5b, and -6 (MAGE-A1-6) simultaneously and detect the specific expression of at least one gene (10). By using this common primer, lung cancer detection rates were 83.3% in the cancer tissue, 70.0% in bronchial washing fluid, and 47.5% in random sputum (17). Recently, there was the first report for the detection rate of MAGE-A1-6 RT-nested PCR in bronchial washing fluid of peripheral lung cancer compare with CT-guided TTNB (18). Kim et al. reported that MAGE RT-nested PCR showed a dramatic increase of sensitivity (67.9%) compared with that of cytology (21.4%). No difference in the sensitivity between MAGE RT-nested PCR (67.9%) and TTNB (73.1%) was reported (18). Although the report of Kim et al. showed good sensitivity and specificity of MAGE RT-nested PCR for peripheral lung cancer detection, their enrolled number of patients was very small, consisting of 28 cancer and 14 control cases. We wish to utilize the MAGE RT-nested PCR system in clinical practice for lung cancer diagnosis.

In our practice, MAGE RT-nested PCR revealed a similar sensitivity of 64.0% in the lung cancer group, whereas the specificity in benign group was low (79.3%). The positive rate of MAGE-A1-6 RT-nested PCR was significantly higher than that of conventional cytology (14.7%). Furthermore, combining both MAGE-A1-6 RT-nested PCR and conventional cytology offered relatively higher sensitivity (72.0%). The detection rate of 74% for tumor less than 3 cm in size could greatly enhance the clinical usefulness of MAGE-A1-6 RT-nested PCR for the diagnosis of early-stage lung cancer.

For other clinical applications, MAGE-A4 expression has been found to have correlation with the size of the primary tumor and regional lymph node involvement (19), and MAGE-A3 expression has associated with advanced tumor and poor outcome (20, 21). However, our results do not cumulatively nor individually explain the association of MAGE-A1-6 expression with advanced tumor. On the contrary, the detection rates of MAGE-A1-6 RT-nested PCR for tumor less than 3 cm tend to be increased compared with that of larger tumors. Another study has also reported no difference of MAGE gene expression between different primary tumors (13).

The expression of MAGE gene usually differs according to tumor histology. Many studies have reported high expression of MAGE-A family members (12-14, 16). However, we did not find the differential expression of MAGE-A1-6 between different tumor histologies. Even if there was no significant difference, our result reveals a higher expression rate of MAGE-A1-6 in adenocarcinoma compared to that of squamous cell carcinoma. A recently published study using bronchial washing fluid also reports no different expression between squamous cell carcinoma and adenocarcinoma (15). Most peripheral lung cancers are adenocarcinomas, hence the detection rate for adenocarcinoma is very important.

We also evaluated the MAGE-RT-nested PCR for benign diseases. The false-positive rate was 20.7%. Most of cases were of pulmonary tuberculosis, tuberculoma and benign granulomatous disease. It demonstrates that MAGE-A gene can occasionally be expressed in severe chronic inflammation. The plausible mechanisms contributing to expression of MAGE in tuberculosis might be alterations derived from the persistence of chronic inflammatory reaction. However, PCR contamination by PCR product cannot be completely ruled out because PCR products were analyzed on an electrophoresis gel.

In our study, we aimed to evaluate the clinical usefulness of MAGE-A1-6 RT-nested PCR of bronchial washing for detection of lung tumors invisible by bronchoscopy. In spite of the relative small amounts of bronchial washing fluid, MAGE-A1-6 RT-nested PCR method had high sensitivity in diagnosis of lung cancer not accessible with bronchoscopy. A possible explanation for this result might be that the MAGE-A1-6 common primer can detect the small number of cancer cells in bronchial washing fluid. Therefore, given an increase of peripheral lung tumor, MAGE-A1-6 RT-nested PCR of bronchial washing fluid could have important clinical significance.

The use of the MAGE-A1-6 RT-nested PCR of bronchial washing specimens provided us with promising data regarding the detection of peripheral lung cancer. We suggest that MAGE-A1-6 RT-nested PCR of bronchial washing fluid may be a potentially useful method to improve the diagnosis of peripheral lung cancer in clinical practice.

**Conflict of Interest**

The Authors have stated explicitly that there are no conflicts of interest in connection with this article.

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